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Protective Role of Angiogenin Against Hematopoietic Syndrome of the Acute Radiation Syndrome

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14. ABSTRACT

We have examined the function of angiogenin (ANG) in radiation-induced cell damage by examining the differential effect of γ-radiation toward WT and Ang-/- mice. We have found that ANG deficiency reduced survival, accompanied by reduced HSPC and LymPro number and more active cycling, but reduced MyePro number with restricted proliferation irradiation. We have also assessed the therapeutic activity of recombinant ANG protein and found that treatment with ANG protein, either prior to or post γ-irradiation, significantly extended mice survival, accompanied by recovery of BM cellularity, leukocyte counts, cell numbers of HSPC, LymPro and MyePro. Significantly, cell cycle status of HSPC and LymPro decreased whereas that of MyePro increased upon ANG treatment in irradiated mice. Together, we have demonstrated the protective and mitigative functions of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of MyePro proliferation.

15. SUBJECT TERMS

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1. Introduction

The hypothesis to be tested in this project was that angiogenin (ANG) has a protective role against hematopoietic syndrome of the acute radiation syndrome (H-ARS) and is able to attenuate the effect of residual bone marrow damage (RBMD) after radiation exposure. The objective of this proposal was to obtain proof-of-principle data that supplemental therapy of ANG protein is a feasible strategy for developing medical countermeasure against H-ARS. We planned to examine the effect of ANG deficiency and overexpression on radio-sensitivity in terms of animal survival, severity of H-ARS, and RBMD. *Ang* knockout and transgenic mice were planned to be used for this purpose. They would be irradiated with different doses of γ-radiation and analyzed for complete blood count (CBC), bone marrow (BM) and peripheral blood (PB) profiles including hematopoietic stem cell (HSC), immature and mature myeloid, lymphoid, and erythroid. Post-irradiation survival would also be recorded and compared with that of mic. The protective and therapeutic activities of ANG against H-ARS would also be examined. For this purpose, recombinant mouse ANG protein would be administered into mice 24 h before, immediate or 24 hours after they receive γ-irradiation, and the effect on animal survival and functional recovery of hematopoiesis would be determined.

2. Keywords

Angiogenin, radio protection, radio mitigation, medical countermeasure for radiation exposure, strategic national stockpile

3. Accomplishments

What were the major goals and objectives of the project?

The major goal is to obtain proof-of-principle data that supplemental therapy of ANG protein is a feasible strategy for developing medical countermeasure against H-ARS. This goal is to be accomplished by 4 major tasks. 1) Determine the effect of ANG levels on post-irradiation survival; 2) Analyze the effect of ANG levels on hematopoietic recovery after radiation exposure; 3) Assess the effect of ANG protein supplemental therapy on post-irradiated animal survival; 4) Examine the effect of ANG protein supplemental therapy on function of hematopoietic system.

What was accomplished under these goals?

We have 1) determined the survival of WT and ANG KO mice after exposure to 7.25 Gy, 7.76 Gy, and 8.25 Gy radiation; 2) analyzed complete blood counts and peripheral blood profiles of WT and ANG KO mice after radiation exposure; 3) examined the beneficial effect of ANG protein supplemental therapy on post-irradiation animal survival; 4) examined the effect of ANG protein supplemental therapy on function of hematopoietic system.

Significant Results:

1) *Ang*$^{-/-}$ mice displayed reduced survival following exposure to various doses of γ-radiation, accompanied by decreased blood leukocyte recovery, reduced total BM cellularity, reduced hematopoietic stem and progenitor cells (HSPC) and lymphoid-restricted progenitor (LymPro) number, and more active cycling. In contrast, myeloid-restricted progenitors (MyePro) in *Ang*$^{-/-}$ mice showed reduced cell number, but restricted proliferation following irradiation.

2) Upon γ-irradiation, *Ang*$^{-/-}$ mice also demonstrated increased apoptosis in all cell types, as well as reduced lymphoid and myeloid colony formation. Together, these data suggest that ANG deficiency leads to reduced animal survival, accompanied by diminished cell number, perturbed cell cycling, and elevated apoptotic activity in hematopoietic cells.

3) Treatment with recombinant ANG protein enhances animal survival. We pretreated WT or *Ang*$^{-/-}$ mice with ANG daily for three successive days and irradiated mice with 8.0 Gy 24 hours following the final ANG treatment. Significantly, the 30-day survival rate increased from 20% to 90% after ANG treatment, indicating that ANG is radioprotective. Importantly, 80% of *Ang*$^{-/-}$ mice also survived following ANG pretreatment whereas 100% of untreated *Ang*$^{-/-}$ mice died. Pre-treatment with ANG protected against radiation (4 Gy)-induced loss of cell number and increase in cycling of HSPC and LymPro. In contrast, ANG pre-treatment not only prevented the loss of PyePro but also promoted their proliferation. Moreover, ANG protected against radiation-induced apoptosis in all cell types, and led to enhanced colony formation and post-transplant reconstitution. Together, these data demonstrate the protective function of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of MyePro proliferation.

4) ANG has therapeutic activity as a radio-mitigating agent. We irradiated mice with 8.0 Gy and began ANG treatment 24 hours later, and found that the majority of ANG-treated mice survived, including ANG-treated *Ang*$^{-/-}$ mice, suggesting that ANG has radio-mitigating capabilities. A similar enhancement of survival was observed when ANG treatment was begun immediately following irradiation. Importantly, treatment with ANG 24 hour post-irradiation prevented TBI-induced reduction of overall BM cellularity, as well as HSPC cells and MyePro.

5) ANG has dichotomous role toward stem and progenitor cells in the hematopoietic system in hematopoietic regeneration after radiation damage. We found that post-irradiation treatment of ANG restricted proliferation of HSPC, and simultaneously enhanced proliferation of MyePro. Further, ANG prevented radiation-induced apoptosis in both HSPC cells and MyePro. Significantly, defects in colony formation and post-transplant reconstitution can be rescued by ANG treatment.

6) We also assessed the protective and mitigative effect of ANG in lethally-irradiated animals and found that ANG treatment either before or after lethal irradiation improved survival, and enhanced BM cellularity, as well as peripheral blood content. Moreover, ANG significantly increased the LD50 when treatment was begun 24 hours post-irradiation. Further, treatment with ANG upregulated pro-self-renewal genes in HSPC cells and led to enhanced pro-survival transcript levels and reduced pro-apoptotic transcripts in both HSPC cells and MyePro. Importantly, ANG treatment enhanced rRNA transcription only in MyePro and tiRNA production only in HSPC cells following radiation, consistent with its dichotomous role in promoting and restricting cell proliferation in these two cell types.

Together, these results establish a model by which ANG simultaneously stimulates proliferation of rapidly-responding myeloid-restricted progenitors and preserves HPSC stemness, in association with enhanced hematopoietic regeneration and improved survival.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

The findings were reported in a paper published in Cell on Aug 11, 2016 (Appendix 1).

> Goncalves, K. A., Silberstein, L., Li, S., Severe, N., Hu, M. G., Yang, H., Scadden, D. T., and Hu, G. F. (2016) Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. Cell 166, 894-906.

Tufts Medical Center also issued a press release on publication of above paper that contains these finding (Appendix 2).

What do you plan to do during the next reporting period to accomplish the goals?

In the remaining period of time covered by this award (5 Aug 2016 – 4 Feb 2017), we will evaluate the effect of ANG overexpression ANG receptor PlexinB2 on radiosensitivity. We will document survival of ANG transgenic mice upon radiation exposure. We will also knockout PlexinB2 in hematopoietic cells and examine the effect on animal survival upon radiation exposure.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Our findings provide a practical application of recombinant ANG for preventing and mitigating radiation exposure in a scenario of catastrophic accident or terrorism. ANG was found to be effective against radiation-induced hematopoietic syndrome and significantly enhanced survival when administered 24 hours either before or after radiation exposure. Currently, there are no FDA-approved drugs to treat severely irradiated individuals. An efficacy requirement mandated by The Radiation and Nuclear Countermeasures Program at the National Institute of Allergy and Infectious Diseases, and ANG apparently satisfies this requirement. Moreover, current standard-of-care approaches, including (G- Neuprogen (G-CSF) and its derivatives, which is on the list of Strategic National Stockpile, target a limited progenitor cell pool and require repeated doses to combat radiation-induced neutropenia. In this regard, ANG is a promising candidate as a medical countermeasure for radiation exposure.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Nothing to report

6. Products

Publications

Goncalves, K. A., Silberstein, L., Li, S., Severe, N., Hu, M. G., Yang, H., Scadden, D. T., and Hu, G. F. (2016) Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells. Cell 166, 894-906.

Patent applications

Hu G.F., Goncalves K., Silberstein L., Scadden D.T. Treatment with angiogenin to enahcne hematopoietic reconstitution. US application number 62/315,281. Filed on 3/30/2016.

7. Participants & other collaborating organizations

What individuals have worked on the project?

Name: Guo-fu Hu
Project Role: Principal Investigator
Nearest person month worked: 2
Contribution to Project: Conceived ideas, designed experiments, interpreted data, and wrote manuscript
Funding Support: This award and NIH

Name: Shuping Li
Project Role: Research Fellow
Nearest person month worked: 6
Contribution to Project: Performed survival analysis, FACS analysis, and transplantation.
Funding Support: This award and NIH

Name: Nil Vanli
Project Role: Graduate Student
Nearest person month worked: 12
Contribution to Project: Prepared ANG proteins, maintained animal clones.
Funding Support: This award

Name: Kevin Goncalves
Project Role: Graduate Student
Nearest person month worked: 12
Contribution to Project: Irradiated mice, bone marrow transplantation.
Funding Support: NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. Special reporting requirements

None

9. Appendices

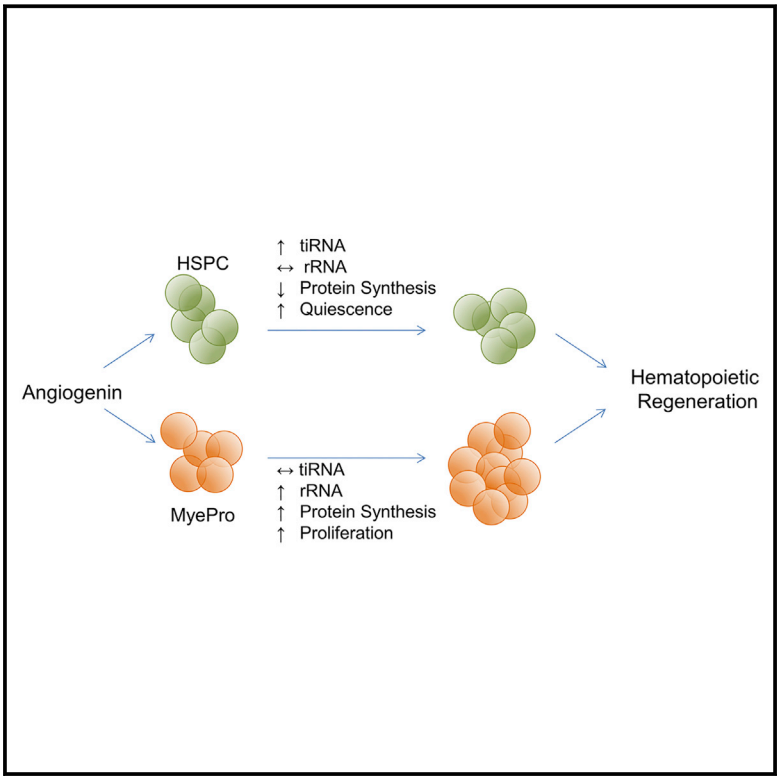
1) Reprint
2) Press release

Article

Cell

Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells

Graphical Abstract



Highlights

- HSPC quiescence and progenitor cell proliferation are simultaneously enhanced by ANG
- The dichotomous effect of ANG is related to differential RNA processing
- ANG prevents and mitigates radiation-induced bone marrow failure
- ANG dramatically improves transplantation efficiency of mouse and human HSPCs

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In Brief

A new niche-specific modulator maintains the stemness of hematopoietic stem/progenitor cells while promoting the proliferation of myeloid progenitor cells through the regulation of RNA metabolism.

Goncalves et al., 2016, Cell *166*, 894–906
August 11, 2016
http://dx.doi.org/10.1016/j.cell.2016.06.042

CellPress

Cell

Article

Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells

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SUMMARY

Regulation of stem and progenitor cell populations is critical in the development, maintenance, and regeneration of tissues. Here, we define a novel mechanism by which a niche-secreted RNase, angiogenin (ANG), distinctively alters the functional characteristics of primitive hematopoietic stem/progenitor cells (HSPCs) compared with lineage-committed myeloid-restricted progenitor (MyePro) cells. Specifically, ANG reduces the proliferative capacity of HSPC while simultaneously increasing proliferation of MyePro cells. Mechanistically, ANG induces cell-type-specific RNA-processing events: tRNA-derived stress-induced small RNA (tiRNA) generation in HSPCs and rRNA induction in MyePro cells, leading to respective reduction and increase in protein synthesis. Recombinant ANG protein improves survival of irradiated animals and enhances hematopoietic regeneration of mouse and human HSPCs in transplantation. Thus, ANG plays a non-cell-autonomous role in regulation of hematopoiesis by simultaneously preserving HSPC stemness and promoting MyePro proliferation. These cell-type-specific functions of ANG suggest considerable therapeutic potential.

INTRODUCTION

A population of quiescent adult stem cells with self-renewal and differentiation capabilities is required for tissue homeostasis and regeneration (Orford and Scadden, 2008). Stem cell quiescence has been shown to protect cells from exhaustion, especially under stress, which is essential for both continuous cell output and prevention of malignant transformation (Nakamura-Ishizu et al., 2014). In the hematopoietic system, this is achieved by both cell-intrinsic and -extrinsic factors. Cell-cycle and epigenetic regulators—as well as pathways involved in growth control, including cyclin-dependent kinases and inhibitors, Rb, PI3K, and p53–have been demonstrated as cell-intrinsic regulators of hematopoietic stem/progenitor cells (HSPCs) proliferation (Ito and Suda, 2014; Nakamura-Ishizu et al., 2014). Various secreted and cell-surface factors that are produced by bone marrow (BM), including angiopoetin-1, thrombopoietin, SCF (stem cell factor), CXCL12, and TGF-β (transforming growth factor β) (Ito and Suda, 2014; Mendelson and Frenette, 2014; Morrison and Scadden, 2014), have been shown to extrinsically regulate HSPCs.

Recent strides have been made to therapeutically harness growth control properties of hematopoietic stem cells (HSCs) in an effort to improve hematopoietic regeneration in the clinic. In the context of HSC transplantation (SCT), in particular, low numbers of HSPCs result in low transplantation efficacy, which can markedly affect the survival of patients undergoing SCT (Smith and Wagner, 2009). Therefore, expanding transplantable cell numbers has been a long-standing goal (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Himburg et al., 2010; North et al., 2007). Preserving HSC function can be at odds with expansion strategies, but advances in improved BM homing (Li et al., 2015) and maintained stemness through protection against extraphysiologic oxygen shock (Mantel et al., 2015) are being made. To our knowledge, however, no studies to date have accomplished preserving HSC regenerative capacity through quiescence while enabling progenitor expansion.

Angiogenin (ANG), also known as RNase5, is a member of the secreted vertebrate-specific RNase superfamily and has angiogenic (Fett et al., 1985), neurogenic (Subramanian and Feng, 2007), neuroprotective (Subramanian et al., 2008), and immune-regulatory (Hooper et al., 2003) functions. Under growth conditions, ANG promotes proliferation and enhances survival in a variety of cell types, including endothelial (Kishimoto et al., 2005), neuronal (Kieran et al., 2008), and cancer (Yoshioka et al., 2006) cells. The growth stimulatory function of ANG is mediated through rRNA transcription (Tsuji et al., 2005) and requires nuclear translocation of ANG (Xu et al., 2003). Under stress, ANG is translocated to stress granules (SGs) and mediates the production of tRNA-derived stress-induced small RNA (tiRNA); these small RNA species enhance cellular survival by simultaneously suppressing global protein translation, saving anabolic energy,

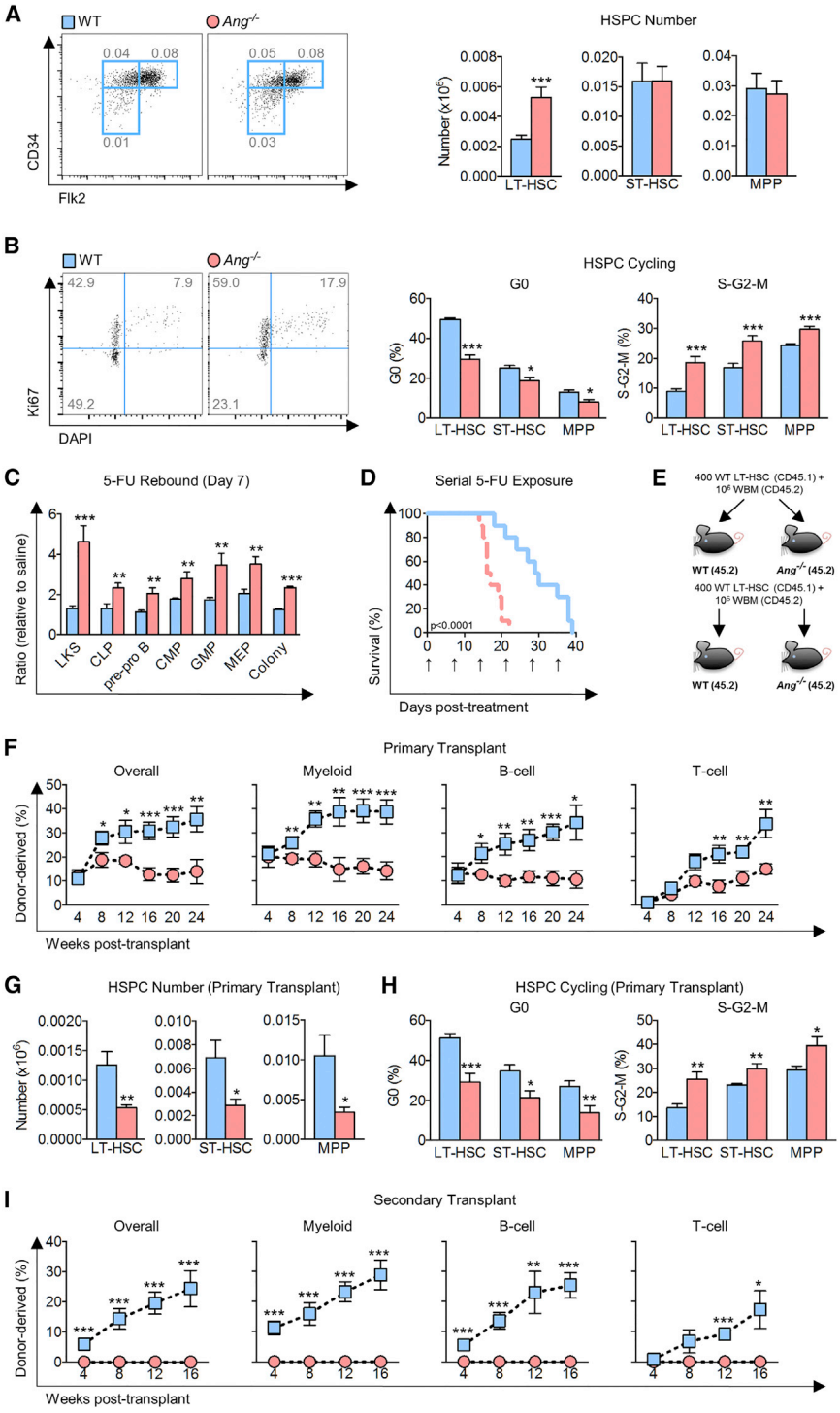

Figure 1. *Ang* Deficiency Results in Loss of HSPC Quiescence and Defective Transplantation

(A and B) Quantification of primitive hematopoietic cell number per femur (A; n = 12) and cell-cycle status (B; n = 8) in $Ang^{-/-}$ mice.

(C) Quantification of stem and progenitor cells in $Ang^{-/-}$ mice on day 7 post-exposure to 150 mg/kg 5-FU (n = 8).

(D) Survival of $Ang^{-/-}$ mice following weekly 5-FU (150 mg/kg) exposure (n = 10). Arrows indicate day of injection.

(E) Experimental schema of serial transplant using WT or $Ang^{-/-}$ hosts.

(F–H) Multi-lineage donor cell chimerism (F), HSPC number per femur (G), and HSPC cell-cycle status (H) after competitive primary transplantation of WT LT-HSCs into lethally irradiated WT or $Ang^{-/-}$ recipients (n = 8).

(I) Chimerism after secondary transplantation of sorted LT-HSCs from primary recipients into WT or $Ang^{-/-}$ secondary recipients (n = 8).

Bar graphs represent mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figure S1 and Tables S1 and S2.

and permitting internal ribosomal entry sequence (IRES)-mediated protein translation of anti-apoptotic genes (Emara et al., 2010; Ivanov et al., 2011; Yamasaki et al., 2009).

In this study, we demonstrate that ANG restricts proliferation of primitive HSPCs but stimulates proliferation of myeloid-restricted progenitor (MyePro) cells. We also demonstrate that ANG mediates tiRNA production in HSPCs but promotes rRNA transcription in MyePro cells. Importantly, these properties of ANG are reflected by enhanced hematopoietic regeneration and animal survival upon treatment with recombinant ANG protein following radiation-induced BM failure and a dramatic increase in the level of hematopoietic reconstitution by ANG-treated mouse LT-HSCs (long-term HSCs) and human $CD34^+$ cord blood (CB) cells. Therefore, ANG is a previously unrecognized regulator of HSPCs, with unique RNA processing function relevant to radiation-induced BM failure and clinical SCT.

RESULTS

ANG Plays a Non-Cell-Autonomous Role in Regulation of LT-HSC Quiescence and Self-Renewal

We sought to examine the functional role of ANG in hematopoiesis because it was originally found to be differentially expressed in BM osteolineage cells in close proximity to transplanted HSPCs (Silberstein et al., 2016). The presence of ANG mRNA in mesenchymal cells from BM was confirmed by qPCR of sorted subsets of cells (Figure S1A). We then profiled HSPCs (Figure S1B) in the BM of *Ang* knockout ($Ang^{-/-}$) mice and found a 2-fold increase in the number of LT-HSCs, but not ST-HSCs (short-term HSCs) or multi-potent progenitors (MPPs), in $Ang^{-/-}$ BM (Figure 1A).

Consistent with this finding, a reduction in G0 phase and a corresponding increase in S/G2/M phases of the cell cycle (Figure 1B), as well as enhanced bromodeoxyuridine (BrdU) incorporation (Figure S1C), were observed in *Ang*$^{-/-}$ LT-HSCs. *Ang*$^{-/-}$ ST-HSCs and MPPs also displayed increased cycling (Figures 1B and S1C) but to a less severe degree (for LT-HSCs, ST-HSCs, and MPPs, respectively, the percentage increases in S/G2/M phases were 107.7 ± 23.7, 52.2 ± 10.0, and 21.9 ± 4.6, and the percent decreases in G0 phase were 40.1 ± 4.3, 24.3 ± 7.0, and 37.6 ± 10.3), which, combined with elevated apoptosis across hematopoietic lineages in *Ang*$^{-/-}$ mice (Figure S1D), might partially explain why no difference in cell number was observed for ST-HSCs and MPPs (Figure 1A). These patterns were also observable by alternative cell-surface markers (SLAM/CD48) for HSPC subtypes (Cabezas-Wallscheid et al., 2014) (Figures S1E and S1F), confirming that *Ang*$^{-/-}$ LT-HSCs cycle more actively.

Despite the significant increase in LT-HSC number in *Ang*$^{-/-}$ BM (Figures 1A and S1E), only mild lymphocytosis was apparent in 8- to 12-week-old mice at baseline (Table S1). However, under conditions of stress, progenitor response to the genotoxic agent, 5-fluorouracil (5-FU), was markedly exaggerated in *Ang*$^{-/-}$ mice (Figure 1C). Further, exposure of these animals to serial proliferative stress, such as weekly injections of 5-FU, resulted in excess animal morality (Figure 1D). Consistent with the phenotype of stress-induced exhaustion (Orford and Scadden, 2008), aged 22-month-old *Ang*$^{-/-}$ mice developed leukopenia (Table S2) and showed a marked reduction in the number of primitive hematopoietic cells in the BM (Figure S1G), accompanied by more active HSPC cycling (Figure S1H). Aged *Ang*$^{-/-}$ mice also displayed reduced functional capabilities by in vitro methylcellulose assays (Figures S1I and S1J) and in vivo competitive transplantation (Figures S1K and S1L).

To further characterize the functional significance of elevated cycling in *Ang*$^{-/-}$ HSPCs, we performed transplant experiments by injecting either sorted LT-HSCs (Figure 1E) or total BM (Figure S1M) into lethally irradiated wild-type (WT) or *Ang*$^{-/-}$ hosts. In both experiments, impaired long-term multi-lineage reconstitution was observed in *Ang*$^{-/-}$ hosts (Figures 1F and S1N), with particularly pronounced impairment at later time points. Notably, WT HSPCs in the ANG-deficient microenvironment displayed a significantly reduced HSPC number, accompanied by more active cycling (Figures 1G and 1H). To rule out a homing defect as a cause of impaired reconstitution in *Ang*$^{-/-}$ hosts, CFSE-labeled CD45.1 Lin^{-} cells were injected into irradiated WT or *Ang*$^{-/-}$ recipients, and no difference in the percentage of LKS cells or MyePro cells in the BM of these animals was observed 16 hr after transplantation (Figure S1O).

In order to evaluate the effect of niche-derived ANG (Silberstein et al., 2016) on HSC self-renewal, we carried out serial transplantation experiments. While competitive transplantation demonstrated no detectable hematopoietic contribution by LT-HSCs that had been passaged through ANG-deficient primary recipients (Figure 1I), non-competitive transplantation of whole BM cells from primary *Ang*$^{-/-}$ recipients resulted in the death of all secondary *Ang*$^{-/-}$ recipients (Figure S1P). The marked inability to reconstitute in both transplant settings indicates severe loss of HSC self-renewal capacity in ANG-deficient hosts. Taken together, these data demonstrate that ANG plays a non-cell-autonomous role in the regulation of quiescence and self-renewal of primitive hematopoietic cells, particularly LT-HSCs.

ANG Enhances MyePro Proliferation

The finding that ANG restricts cell cycling of HSPCs is the first known evidence for a suppressive activity of ANG on cell proliferation, as all previous studies revealed that ANG promotes cell proliferation (Li and Hu, 2010). Therefore, we examined cell-type-specific effects of ANG in various cells of the hematopoietic lineage. We observed that, while *Ang*$^{-/-}$ LKS cells cycle more actively, *Ang*$^{-/-}$ MyePro cells showed reduced cycling by Ki67 (Figure 2A) and BrdU (Figure S2A) staining.

The cell-type specificity of ANG was further illustrated by analyzing lymphoid-restricted progenitor (LymPro) cells and MyePro cells, including common lymphoid progenitor (CLP), pre-pro B, common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP) cell types. As with HSPCs, we found that *Ang*$^{-/-}$ CLPs and pre-pro B cells (Figure S2B) cycle more actively (Figure S2C) and incorporate more BrdU (Figure S2D), suggesting that ANG restricts LymPro proliferation. In contrast, *Ang*$^{-/-}$ MyePro cells, including CMPs, GMPs, and MEPs, displayed less active cycling (Figure S2F) and reduced BrdU incorporation (Figure S2G), accompanied by a reduction of CMP and GMP numbers (Figure S2E). Importantly, we observed restricted proliferation of myeloid-biased MPP3s and more active cycling of lymphoid-biased MPP4s (Cabezas-Wallscheid et al., 2014) in *Ang*$^{-/-}$ mice (Figure 2B). Together, these data indicate that the function of ANG is cell-type specific: while ANG restricts cell proliferation in primitive HSPCs and LymPro cells, it promotes proliferation of MyePro cells. This cell-type specificity is observable within the earliest phenotypically defined lineage-biased progenitor cell types: MPP3 and MPP4.

Cell-type-specific regulation of ANG was confirmed by the fact that *Ang* deletion resulted in decreased expression of cycle checkpoint or self-renewal genes, including *p21*, *p27*, *p57*, *GATA3*, *vWF*, and *Bmi1* (Cheng et al., 2000; Frelin et al., 2013; Kent et al., 2009; Matsumoto et al., 2011; Park et al., 2003) in LKS cells but not in MyePro cells (Figure S2H). In contrast, the cell-cycle-related gene, cyclin D1, was decreased in MyePro cells, but not in LKS cells, upon *Ang* deletion (Figure S2H). We then examined the effect of recombinant ANG protein on cultured HSPCs and MyePro cells. Remarkably, culture with ANG for 2 hr in PBS led to a dose-dependent increase in the expression of pro-self-renewal genes in LKS cells (Figure 2C). No such change was noted in MyePro cells. In contrast, cyclin D1 was enhanced by culture with ANG in MyePro cells but not in LKS cells (Figure 2C). A similar pattern was observed in LT-HSCs cultured with ANG for 2 hr in PBS (Figure S2I) or under longer culture conditions in cytokine-supplemented S-clone media (Figure S2J). Notably, addition of exogenous ANG led to elevated levels of pro-self-renewal genes in *Ang*$^{-/-}$ LT-HSCs, as was seen in WT cells (Figure S2K). Together, these data demonstrate that ANG differentially regulates gene expression in HSPCs and MyePro cells, including genes relevant for proliferation and self-renewal.

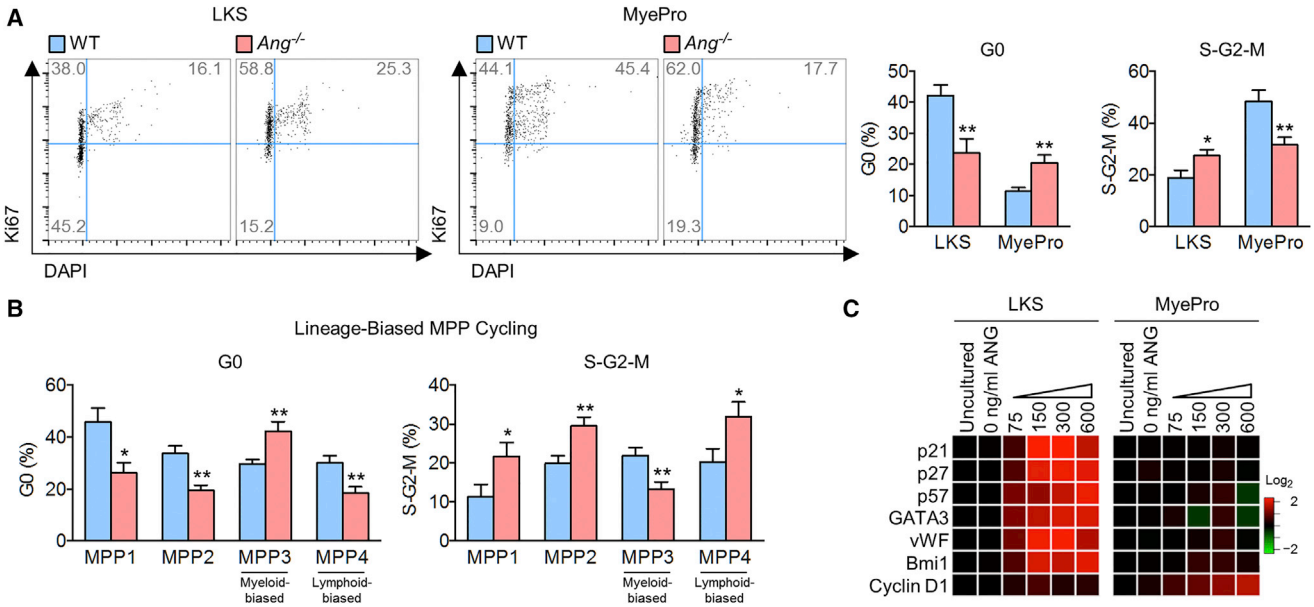

Figure 2. Dichotomous Effect of ANG in LKS and MyePro Cycling

(A and B) Cell-cycle status of LKS cells and MyePro cells (A; n = 8) and of MPP1-4 cells (B; n = 6) in *Ang*$^{-/-}$ mice.

(C) qRT-PCR analysis of pro-self-renewal transcripts from sorted LKS cells or MyePro cells treated with mouse ANG protein (0–600 ng/ml; n = 6).

Bar graphs represent mean ± SEM, and heatmaps represent mean. *p < 0.05; **p < 0.01.

See also Figure S2.

ANG Dichotomously Regulates Protein Synthesis in LKS and MyePro Cells

ANG has been shown in other cell types to regulate global protein synthesis, a housekeeping function recently shown to be tightly regulated in primitive HSCs (Signer et al., 2014). To determine whether ANG regulates protein synthesis in HSPCs, we assessed in vivo protein synthesis in *Ang*$^{-/-}$ mice by a fluorogenic assay using O-propargyl-puromycin (OP-Puro) (Signer et al., 2014). Consistent with their cell-cycle profile (Figures 2A and S2A), *Ang*$^{-/-}$ LKS cells showed a higher rate of protein synthesis, while *Ang*$^{-/-}$ MyePro cells demonstrated reduced protein synthesis (Figure 3A). This cell-type specificity was also evident when BM was analyzed with more specific hematopoietic cell markers (Figure S3A) or when assessing cells in G0/G1 phase (Figure S3B). In vivo administration of OP-Puro did not alter BM cellularity or LT-HSC frequency (Figures S3C and S3D). Significantly, in vitro culture of LKS cells with ANG led to reduced protein synthesis, while the addition of ANG to MyePro cells led to enhanced protein synthesis (Figure 3B). Together, these data demonstrate that the effect of ANG on protein synthesis is cell-type specific.

The Restrictive Function of ANG in HSPCs Is Mediated by tiRNA

To reveal the biochemical mechanism for this dichotomous effect of ANG on protein synthesis, we first assessed rRNA transcription, which is stimulated by ANG in other cell types (Kishimoto et al., 2005; Tsuji et al., 2005). The addition of ANG led to enhanced rRNA transcription in MyePro and whole BM cells, but not in LKS cells (Figure 3C), while *Ang* deletion resulted in reduced rRNA transcription in MyePro and whole BM cells but not in LT-HSCs (Figure S3E). These findings are consistent with increased proliferation and protein synthesis observed in MyePro cells following ANG treatment.

ANG has been shown to reprogram protein synthesis as a stress response to promote survival under adverse conditions. This function of ANG is mediated by tiRNA, a noncoding small RNA that specifically permits translation of anti-apoptosis genes while global protein translation is suppressed so that stressed cells have adequate time and energy to repair damage, collectively promoting cell survival (Emara et al., 2010; Ivanov et al., 2011; Yamasaki et al., 2009). To assess whether ANG-mediated regulation of protein synthesis is tiRNA dependent, we assessed bulk small RNA production by electrophoresis. LKS cells exhibited dramatically higher small RNA production than MyePro cells at baseline (Figure 4A). tiRNA level was normally low in Lin^{+}-differentiated cell types, although it became detectable when a higher amount (15 μg) of total RNA was assessed (Figure S3F). Importantly, the addition of ANG led to markedly elevated tiRNA levels in LKS cells (Figure 4A). Equal loading was affirmed by tRNA levels (indicated by arrows in Figure 4A). The addition of ANG to Lin^{+} cells did not result in an increase in tiRNA levels, in contrast to significantly elevated tiRNA levels following ANG treatment of HSPCs (Figure S3F; compare to Figure 4A). Furthermore, tiRNA levels in *Ang*$^{-/-}$ LKS cells were substantially reduced but not completely diminished (Figure S3G). As ANG is the only RNase that has been demonstrated to mediate tiRNA production (Yamasaki et al., 2009), this finding suggests that other unidentified RNases may be responsible for the remaining level (29%) of tiRNA. Moreover, we also observed

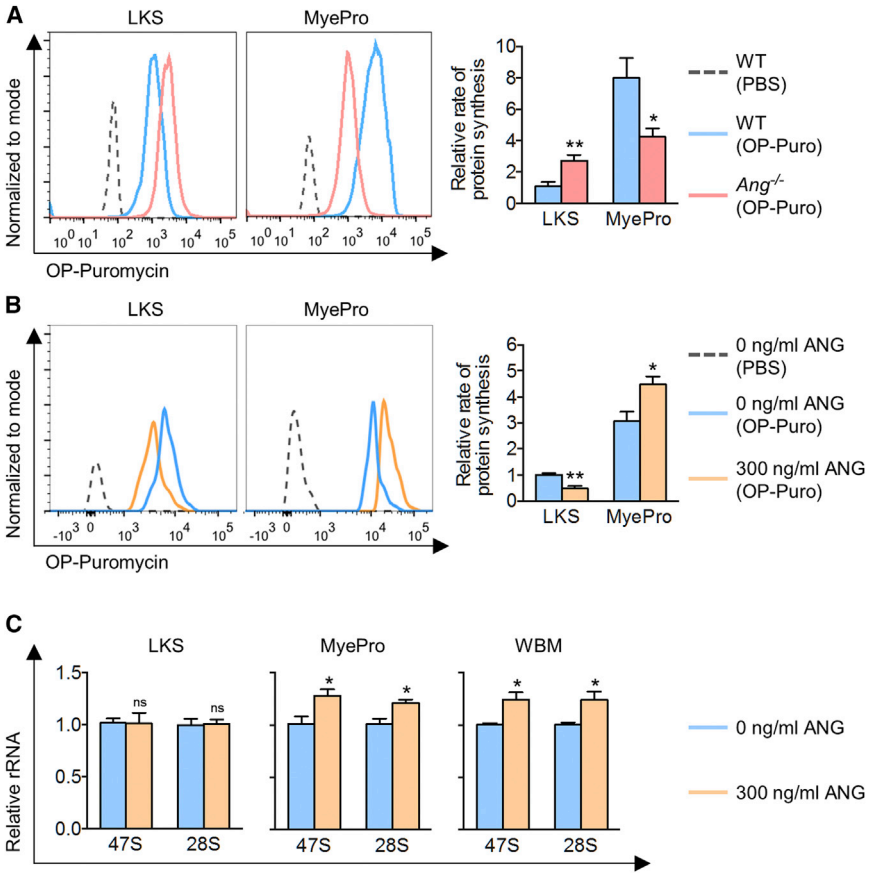

Figure 3. ANG-Mediated Regulation of Protein Synthesis Is Cell-Type Specific

(A) In vivo OP-Puro incorporation in WT or *Ang*$^{-/-}$ LKS cells and MyePro cells. Cells were analyzed 1 hr after OP-Puro administration. Bar graphs indicate relative values to WT LKS (n = 5).

(B) In vitro OP-Puro incorporation following 2-hr ANG treatment of LKS and MyePro cells. Bar graphs indicate relative values to untreated LKS (n = 6).

(C) qRT-PCR analysis of rRNA with primers targeting mature and precursor transcripts following 2-hr ANG treatment of LKS and MyePro cells, using various primer sets (n = 3). Bar graphs indicate relative values to untreated cells.

Error bars indicate mean ± SEM. *p < 0.05; **p < 0.01; ns, not significant.

See also Figures S3 and S4.

an increase in tiRNA production in MyePro cells by sodium arsenite (SA), which was suppressed by exogenous ANG protein (Figure S3H). In contrast, tiRNA production in LKS cells was not increased by SA but was enhanced by ANG both at steady-state and under SA-induced oxidative stress conditions (Figure S3H). These results demonstrate that ANG differentially regulates tiRNA in LKS and MyePro cells under both homeostatic and stress conditions.

To ensure that bulk small RNA reflect tiRNA, we analyzed the levels of a representative tiRNA, tiRNA-Gly-CCC, by northern blotting in ANG-treated LKS and MyePro cells. tiRNA-Gly-GCC was previously shown to be expressed in hematopoietic tissues, including BM and spleen, but was neither examined in primitive hematopoietic cells nor functionally validated (Dhahbi et al., 2013). Figure 4B shows that tiRNA-Gly-GCC was significantly elevated in LKS cells, relative to MyePro cells, and was further enhanced by exogenous ANG. Together, these data identify tiRNA as a distinct RNA species that is abundantly expressed in HSPCs and that is regulated by ANG.

To determine whether tiRNA was responsible for restricted protein synthesis in HSPCs, we transfected synthetic tiRNA-Gly-GCC in LKS and MyePro cells and assessed protein synthesis in vitro using OP-Puro. As tiRNA requires its 5′-phosphate to suppress protein synthesis (Ivanov et al., 2011), we used an inactive, dephosphorylated synthetic tiRNA-Gly-GCC, termed (d)5′-P-tiRNA, as a negative control. Expectedly, transfection of active 5′-P tiRNA, but not inactive (d) 5′-P-tiRNA, led to a significant reduction in the rate of protein synthesis in both LKS and MyePro cells (Figure 4C). Thus, tiRNA transfection phenocopies exogenous ANG on restriction of protein synthesis in LKS cells, which has been shown in Figure 3B. We also found that myeloid and lymphoid progenitor colony formation was restricted upon transfection of whole BM with active 5′-P tiRNA but not inactive (d)5′-P-tiRNA (Figure S3I). Moreover, transfection of active 5′-P-tiRNA led to upregulation of pro-self-renewal and pro-survival genes, and downregulation of pro-apoptotic genes, in both LKS and MyePro cells by lipofection (Figure 4D) and electroporation (Figure S3J).

The exact subcellular compartment where tiRNA is produced by ANG is currently unknown (Saikia et al., 2014), but the growth and survival function of ANG has been correlated to its SG localization in stressed cells (Pizzo et al., 2013). The finding that ANG produces tiRNA and restricts protein synthesis only in LKS cells prompted us to examine differential localization of ANG in SGs between LKS and MyePro cells. ANG is known to be internalized through receptor-mediated endocytosis and translocated to either the nucleus or SGs, depending on cell state, to mediate rRNA or tiRNA production, respectively (Pizzo et al., 2013). We found that ANG was colocalized with poly(A)-binding protein (PABP), an SG marker, in LKS cells but not in MyePro cells (Figure S4A). Furthermore, we found that RNase/ANG inhibitor 1 (RNH1)—an endogenous ANG inhibitor that has been shown to regulate the subcellular localization of ANG (Pizzo et al., 2013) and that is expressed in BM cell subsets under steady-state conditions (Figure S4B)—is localized in SGs in MyePro cells but not in LKS cells (Figure S4C). This opposing localization pattern of RNH1 and ANG was further examined by double immunofluorescence (Figure S4D) and fluorescence resonance energy transfer (FRET; Figure S4E), which showed that ANG and RNH1 colocalize and interact in the nucleus, but not in the

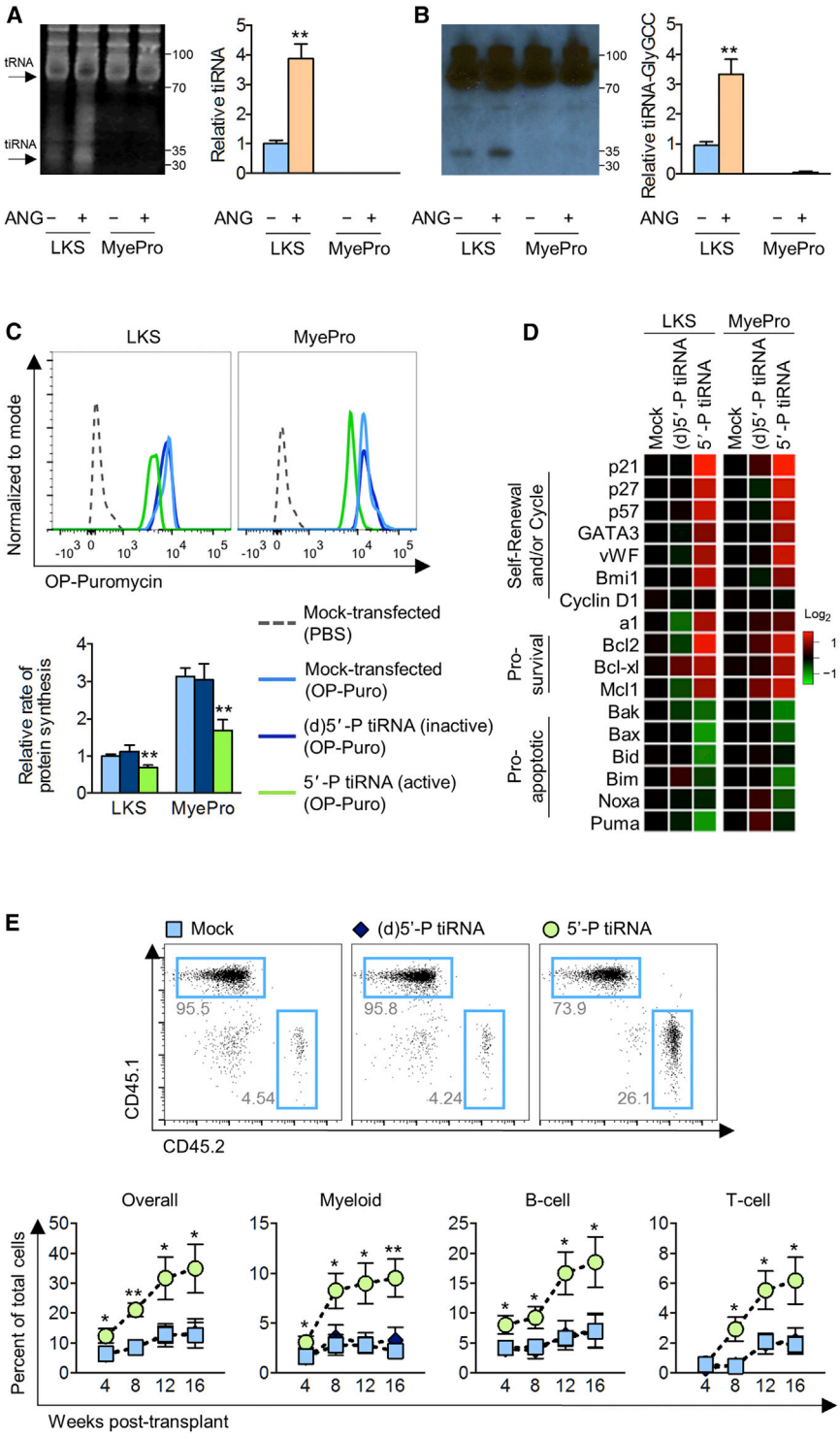

Figure 4. ANG-Mediated Regulation of Protein Synthesis Is Correlated with Cell-Type-Specific tiRNA Production

(A and B) Small RNA production (A; n = 3) and northern blot analysis of tiRNA-Gly-GCC (B; n = 3) following 2-hr treatment of LKS and MyePro cells with ANG. Bar graphs indicate relative values to untreated LKS.

(C and D) OP-Puro incorporation (C; n = 5), and qRT-PCR analysis of pro-self-renewal, pro-survival, and pro-apoptotic transcripts (D; n = 5) in LKS and MyePro cells transfected with inactive (d)5′-P tiRNA or active 5′-P tiRNA.

(E) Post-transplant reconstitution of LKS cells transfected with inactive (d)5′-P tiRNA or active 5′-P tiRNA (n = 7).

Error bars indicate mean ± SEM, and heatmaps represent mean. *p < 0.05; **p < 0.01.

See also Figures S3 and S4.

cytoplasm, of LKS cells and in the cytoplasm, but not in the nucleus, of MyePro cells. Thus, RNH1, which is known to stoichiometrically inhibit ANG with a femtomolar dissociation constant, K_d (Lee et al., 1989), likely inhibits nuclear ANG but not cytoplasmic ANG in LKS cells, suppressing rRNA production but permitting tiRNA production, whereas it inhibits cytoplasmic ANG, but not nuclear ANG, in MyePro cells to allow rRNA transcription but not tiRNA production.

To assess whether tiRNA-mediated regulation of protein synthesis affects HSPC function, we transfected LKS cells with synthetic tiRNA and competitively transplanted those cells into WT hosts. We observed significantly enhanced long-term multi-lineage post-transplant reconstitution of cells transfected with active 5′-P-tiRNA, relative to untreated LKS cells or cells transfected with inactive (d)5′-P-tiRNA (Figure 4E). As ANG stimulates tiRNA production in LKS cells, these data strongly suggest that ANG may enhance the regenerative potential of HSPCs by tiRNA-mediated alterations of protein synthesis.

ANG Is a Pro-regenerative Factor after Radiation-Induced Damage

To assess the pro-regenerative role of ANG, we first examined the function of ANG in the context of radiation-induced cell damage. We found that *Ang* mRNA levels were elevated in mixed niche cells 1 day post-irradiation (Figure S5A), but not in whole BM cells (Figure S5B) or sorted LKS and MyePro cells (Figure S5C). Consistent with this finding, ANG serum levels were also significantly elevated (Figure S5D), suggesting that niche cells increase ANG synthesis as a response to irradiation. *Ang*$^{-/-}$ mice displayed reduced survival following exposure to various doses of γ radiation (Figure S5E), accompanied by reduced blood leukocyte recovery (Figure S5F). When animals were analyzed

at day 7 post-irradiation (Figure S5G), we observed reduced total BM cellularity (Figure S5H; Table S3), reduced HSPC and LymPro cell numbers (Figures S5I and S5K), and more active cycling (Figures S5J and S5L). These data are consistent with the quiescence-inducing effect of ANG on HSPCs and LymPro cells. In contrast, *Ang*$^{-/-}$ MyePro cells showed reduced cell number (Figure S5M) but restricted proliferation (Figure S5N) following γ irradiation. Importantly, this dichotomous regulation of proliferation was observed in lineage-biased MPP3 and MPP4 cells under radiation stress (Figures S5O and S5P), as observed at steady state (Figure 2B). *Ang*$^{-/-}$ mice also demonstrated increased apoptosis in all primitive hematopoietic cell types examined (Figure S5Q), as well as reduced lymphoid and myeloid colony formation in response to γ irradiation (Figure S5R).

To determine whether treatment with ANG enhances survival, we pretreated WT or *Ang*$^{-/-}$ mice with ANG daily for 3 successive days and irradiated mice with 8.0 Gy 24 hr following the final ANG treatment (Figure S6A). Significantly, the 30-day survival rate increased from 20% to 90% after ANG treatment, indicating that ANG is radioprotective (Figure 5A). Importantly, 80% of *Ang*$^{-/-}$ mice also survived following ANG pretreatment whereas 100% of untreated *Ang*$^{-/-}$ mice died. These results indicate that both endogenous and exogenous ANG are radioprotective. Pretreatment with ANG protected against radiation-induced loss of the total BM number (Figure S6B) and various BM cell subsets (Table S4) and restricted HSPC and LymPro cell cycling (Figures S6C and S6D). In contrast, ANG pre-treatment not only prevented radiation-induced loss of MyePro cell number (Table S4) but also further promoted their proliferation beyond those enhanced by γ irradiation (Figure S6E). Moreover, ANG protected against irradiation-induced apoptosis in all primitive hematopoietic cell types examined (Figure S6F) and led to enhanced colony formation (Figure S6G) and post-transplant reconstitution (Figure S6H). Together, these data demonstrate the protective function of ANG against radiation-induced BM damage, likely through induction of HSPC quiescence and promotion of MyePro cell proliferation.

To assess a potential therapeutic use of ANG as a radio-mitigating agent, we irradiated mice with 8.0 Gy and immediately began ANG treatment (Figure S6I), and we observed enhanced survival in ANG-treated mice (Figure S6J). We also irradiated mice with 8.0 Gy and began ANG treatment 24 hr later (Figure S6K). Significantly, the majority of ANG-treated mice survived, including ANG-treated *Ang*$^{-/-}$ mice, suggesting that ANG has radio-mitigating capabilities (Figure 5B). Importantly, treatment with ANG 24 hr post-irradiation prevented radiation-induced reduction of overall BM cellularity (Figure 5C), as well as that of LKS cells, MyePro cells (Figures 5C and 5D), and other BM cells (Table S4). Consistent with its dichotomous role in cell-cycle kinetics, ANG restricted the proliferation of LKS cells and simultaneously enhanced proliferation of MyePro cells under radiation stress (Figure 5E). Furthermore, ANG prevented radiation-induced apoptosis in both cell types (Figure 5F). These effects on cell number, cycling, and apoptosis were also apparent in further defined stem and progenitor cell populations (Figures S6L–S6O; Table S4). Significantly, defects in colony formation (Figure S6P) and post-transplant reconstitution (Figure 5G) could be rescued by ANG treatment.

We also assessed the protective and mitigative effects of ANG in lethally irradiated (12.0-Gy) animals and found that ANG treatment either before or after lethal irradiation improved survival (Figure 5H) and enhanced BM cellularity (Figure 5I) as well as peripheral blood content (Table S5). Consistent with these findings, ANG significantly increased the LD_{50} (lethal dose 50) when treatment was begun 24 hr post-irradiation (Figure 5J). Furthermore, treatment with ANG upregulated pro-self-renewal genes in LKS cells and led to enhanced pro-survival transcript levels and reduced pro-apoptotic transcripts in both LKS cells and MyePro cells under radiation stress (Figure 5K). ANG treatment enhanced rRNA transcription only in MyePro cells, both before and after irradiation (Figure 5K). In LKS cells, ANG did not promote rRNA transcription (Figure 5K) but enhanced tiRNA production under radiation stress (Figure 5L), as has been observed under steady-state (Figure 4A) and under oxidative stress conditions (Figure S3H). These results indicate that the dichotomous role of ANG in regulating proliferation of HSPCs and MyePro cells is preserved under stress conditions. Together, these results establish a model by which ANG simultaneously stimulates proliferation of rapidly responding MyePro cells and preserves HPSC stemness, in association with enhanced hematopoietic regeneration and improved survival.

Ex Vivo Treatment of LT-HSCs with Recombinant ANG Enhances Post-transplant Reconstitution

The in vitro (Figure 2C; Figures S2H–S2K) and in vivo (Figure 5; Figures S5 and S6) activity of ANG in preserving HSPC stemness and in enhancing regeneration prompted us to assess its capacity in improving SCT and its potential for clinical development. Treatment of LT-HSCs with ANG in culture for 7 days led to a dose-dependent decrease of cell proliferation in WT and *Ang*$^{-/-}$ cells (Figure 6A), consistent with its ability to restrict HSPC proliferation. Notably, LKS cells cultured in the absence of ANG resulted in a reduction of tiRNA expression relative to uncultured cells (Figure 6B). In contrast, cells cultured in the presence of ANG not only maintained baseline tiRNA levels but also their responsiveness to further ANG treatment (Figure 6B).

To test whether restriction of proliferation would enhance transplantation efficiency, we competitively transplanted LT-HSCs that were either freshly isolated or had been cultured for 2 hr with or without 300 ng/ml ANG (Figure S7A), the physiological concentration of both mouse (Figure S5D) and human (Yoshioka et al., 2006) ANG. Significantly, we observed that a 2-hr treatment with ANG led to a dramatic increase in multi-lineage post-transplant reconstitution over 24 weeks (Figure 6C). We also found that improved transplant efficiency was observed with LT-HSCs cultured with ANG for 7 days (Figure S7B). Enhanced regeneration was observed over 16 weeks upon secondary transplant without further ANG treatment (Figure 6D), and elevated peripheral blood counts were observed 1 year post-transplant without any indication of leukemia development (Table S6). Significantly, removal of ANG from the media after 7 days in culture did not induce proliferation (Figure S7C), and enhanced levels of pro-self-renewal transcripts were retained (Figure S7D). Treatment with ANG had no effect on homing (Figure S7E). Importantly, treatment of *Ang*$^{-/-}$ LT-HSCs with exogenous ANG ameliorated post-transplant reconstitution defect of

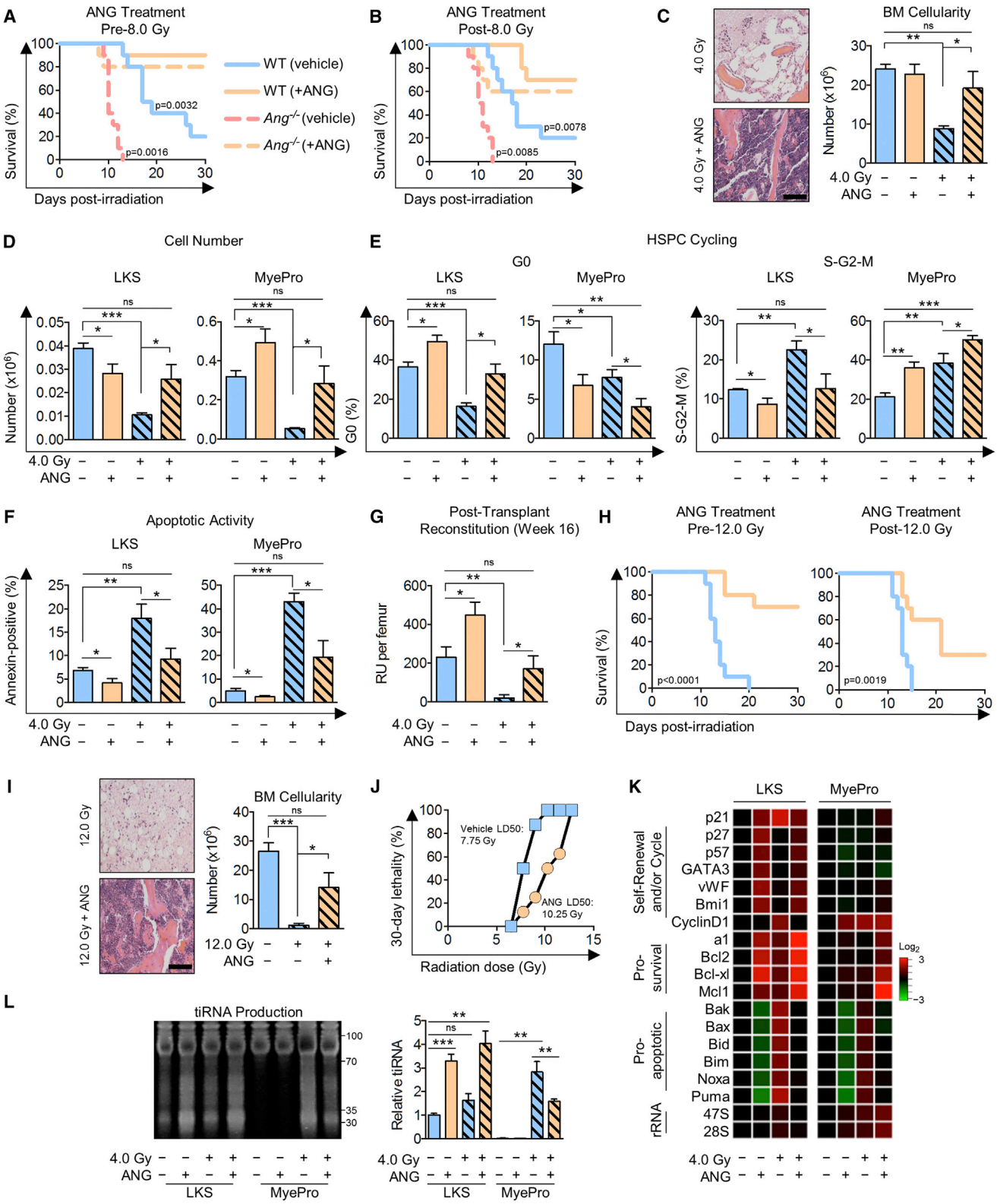

Figure 5. ANG Enhances Radioprotection and Radioresistance

(A) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for 3 successive days 24 hr pre-8.0 Gy (n = 10).

(B) Survival of WT or *Ang*$^{-/-}$ mice treated with ANG daily for 3 successive days 24 hr post-8.0 Gy (n = 10).

(legend continued on next page)

Ang$^{-/-}$ cells and led to enhanced reconstitution over WT cells by week 16 (Figure 6E). Together, these data demonstrate that treatment of LT-HSCs with exogenous ANG significantly enhances their regenerative capabilities upon relatively short exposure, and this effect is long lasting.

ANG Improves Regeneration of Human Cells

Given that ANG significantly improved transplantation efficiency of mouse LT-HSCs, we examined whether human ANG has similar pro-regenerative capabilities in human CD34$^+$ CB cells. Consistent with the anti-proliferative effect of ANG on mouse LT-HSCs, treatment with human ANG led to a dose-dependent reduction of CD34$^+$ CB-cell proliferation over 7 days (Figure 7A) and an elevated level of pro-self-renewal transcripts (Figure S7F), whereas ANG variants that are defective in its ribonucleolytic activity (K40Q) or in receptor binding (R70A) (Hallahan et al., 1991) were inactive (Figures 7A and S7F). Interestingly, R33A ANG, despite having a defective nuclear localization sequence (Moroianu and Riordan, 1994), recapitulated the effect of WT ANG in restricting proliferation (Figure 7A) and enhancing self-renewal signature (Figures 7B and S7F). It is significant to note that a 2-hr exposure to human ANG is adequate for CD34$^+$ human CB cells to upregulate pro-self-renewal genes (Figure 7B), which greatly enhances the translational capability of ANG in improving SCT. The fact that the R33A ANG variant is as active as WT ANG points to the dispensable role of nuclear ANG in HSPCs and reinforces the finding that cytoplasmic localization of ANG is important in preservation of HSPC stemness. Furthermore, ANG treatment of CB cells led to slightly elevated numbers of primitive colonies (Figure S7G). Together, these data indicate that in vitro properties of mouse ANG faithfully translate in a human setting and suggest that the cellular mechanisms underlying mouse HSC regeneration may also translate into human cells.

To assess whether ANG improves transplantation efficiency of human cells, we transplanted CD34$^+$ CB cells that had been cultured for 2 hr in the presence or absence of ANG into NSG (NOD scid gamma) mice at limit dilution and found that treatment with ANG led to elevated frequencies of human CD45$^+$ cells across all doses examined in BM at 16 weeks post-transplant (Figure 7C). Importantly, enhanced regeneration was multi-lineage, as confirmed by the presence of both CD19 B-lymphoid cells and CD33 myeloid cells in BM (Figures S7H and S7I). Remarkably, calculated LT-HSC frequency was 8.9-fold higher in ANG-treated human CD34$^+$ CB cells relative to untreated cells (Figure 7D). Further, enhanced reconstitution was observed upon secondary transplant without further ANG treatment (Figure 7E). These data highlight the translational capacity of ANG in improved transplantation efficacy of clinically relevant human cells.

DISCUSSION

Our study highlights several important findings. First, ANG has a cell-type-specific role in regulating proliferation of HSPCs versus MyePro cells: while promoting quiescence in the former, ANG stimulates proliferation in the latter. Second, we identified a novel RNA-based mechanism by which hematopoiesis is regulated. Importantly, ANG promotes tiRNA production in LKS cells, in association with enhanced stemness in vitro and in vivo. We also show that increased tiRNA production results in reduced levels of global protein synthesis in HSPCs. In contrast, ANG stimulates rRNA transcription in MyePro cells, but not in HSPCs, leading to increased protein synthesis and proliferation. To our knowledge, this is the first report of cell-type specificity in RNA processing that leads to, or originates from, a different cellular state. How cell differentiation state results in the very distinctive effects of ANG is intriguing and of practical consequence, as demonstrated by our studies. Defining the basis for this cell-type specificity is beyond the scope of this report but will be of particular interest for how exogenous signals and intrinsic properties may induce distinctive RNA processing in specific cell types in response to ANG.

Our findings are important, given recent reports demonstrating tight regulation of protein synthesis in HSPCs (Signer et al., 2014). Furthermore, a number of mutations or defects in ribosome function or protein synthesis have been shown to either promote or resist malignant hematopoiesis (Narla and Ebert, 2010). To our knowledge, however, no factors have been shown to link the regulation of HSPC quiescence at the level of protein synthesis. Moreover, a potential therapeutic benefit of using these properties to promote hematopoietic regeneration has not been explored. Modulating tiRNA to alter protein synthesis and cell fate is unique among prior reports of regulatory mechanisms and is of particular interest because of its ability to be affected by a cell-exogenous source. The notion that tiRNA can be cell-state specific in regulating hematopoiesis offers the possibility that similar distinct mechanisms may apply to other tissue types. Discerning whether this is the case and how they may induce altered cell characteristics will help define whether tiRNA represents a common regulatory lever in mammalian biology.

Third, we demonstrate two potential therapeutic uses for ANG. We found that recombinant ANG recapitulates the growth-suppressive properties in vitro and can remarkably improve post-transplant reconstitution of mouse LT-HSCs and human CD34$^+$ CB cells in vivo. Previous studies have identified numerous factors that expand stem cell number in vitro and in vivo (Boitano et al., 2010; Delaney et al., 2010; Fares et al., 2014; Himburg

(C–G) H&E and BM cellularity of femurs (C), LKS and MyePro cell number per femur (D), cell cycling (E), apoptotic activity (F), and post-transplant reconstitution (G) of WT mice treated with ANG daily for 3 successive days 24 hr post-4.0 Gy (n = 6). Scale bar, 100 μm.

(H) Survival of WT mice treated with ANG daily for three successive days 24 hr prior or post-12.0 Gy.

(I) H&E and BM cellularity of femurs of WT mice treated with ANG daily for 3 successive days 24 hr post-12.0 Gy total body irradiation (TBI) (n = 6). Scale bar, 100 μm.

(J) LD_{50} of mice treated with ANG daily for 3 successive days beginning 24 hr post-TBI (n = 8).

(K and L) qRT-PCR analysis of pro-self-renewal, pro-survival, pro-apoptotic, and rRNA transcripts (K; n = 6) and tiRNA production (L; n = 3) in LKS or MyePro cells sorted from irradiated mice (4.0 Gy) and treated with 300 ng/ml ANG.

*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

See also Figures S5 and S6 and Tables S3–S5.

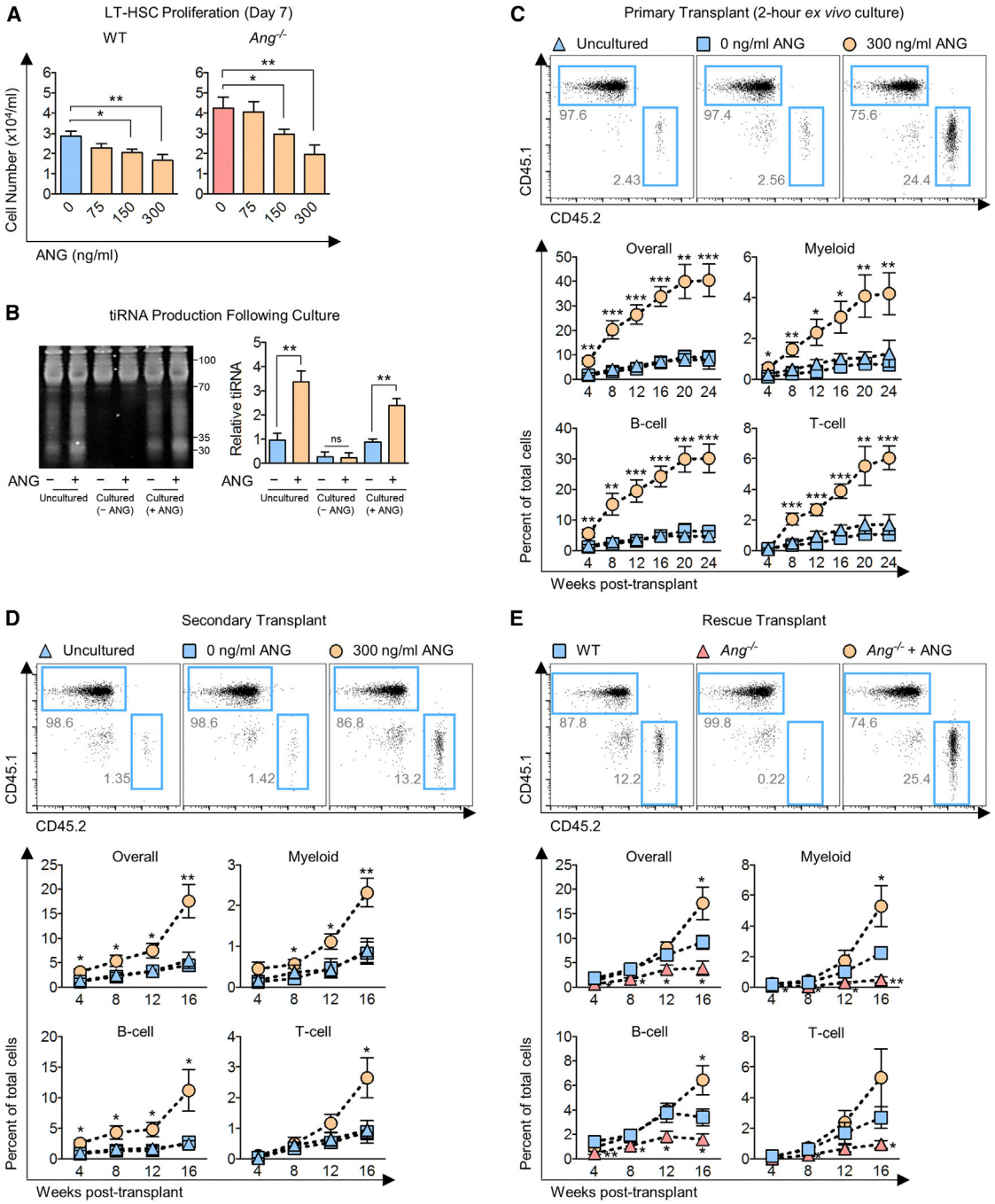

Figure 6. ANG Enhances Post-transplant Reconstitution

(A) Cell density on day 7 from sorted WT or *Ang*$^{-/-}$ LT-HSCs (1,875 cells per milliliter) cultured in the presence of various doses of ANG (n = 6).

(B) tiRNA levels following 7-day culture with 0 or 300 ng/ml ANG. After culture, cells were harvested and again treated with 0 or 300 ng/ml ANG (indicated by + or −) for 2 hr prior to analysis by electrophoresis (n = 3).

(C) Post-transplant reconstitution of WT LT-HSCs (CD45.2) after 2-hr ex vivo treatment with ANG (n = 8–9).

(D) Secondary transplant of WT LT-HSCs (CD45.2) without further ex vivo ANG treatment (n = 7–8).

(E) Post-transplant reconstitution of WT or *Ang*$^{-/-}$ LT-HSCs that were cultured in the presence or absence of 300 ng/ml ANG for 2 hr and competitively transplanted in WT hosts (n = 7).

Error bars indicate mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

See also Figure S7 and Table S6.

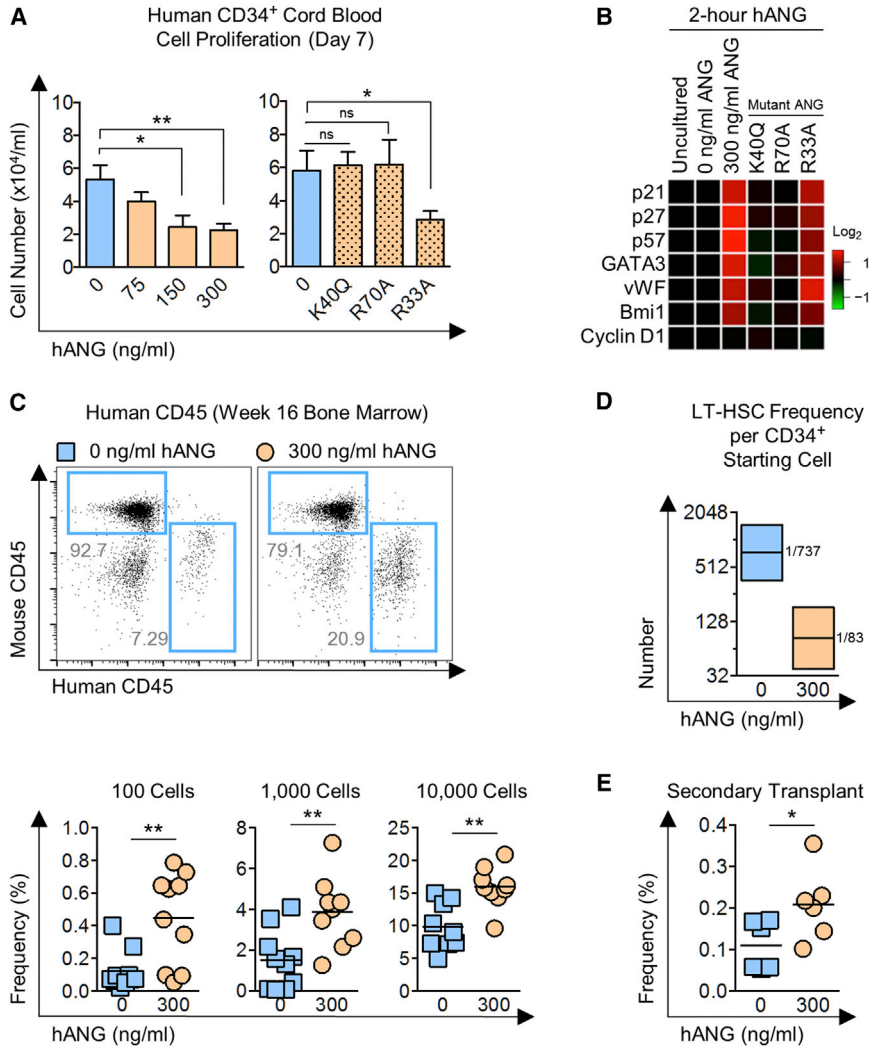

Figure 7. ANG Enhances Post-transplant Reconstitution of Human CD34⁺ CB Cells

(A) Cell number on day 7 from human CD34⁺ CB cells (2,500 cells per milliliter) cultured in the presence of various doses of ANG or ANG variants: K40Q (enzymatic variant), R70A (receptor-binding variant), or R33A (nuclear localization variant) at 300 ng/ml (n = 6). hANG, human ANG protein.

(B) qRT-PCR analysis of pro-self-renewal transcripts in human CD34⁺ CB cells following 2 hr culture with 300 ng/ml human ANG protein (n = 6).

(C) Human CD45 cells in BM of NSG mice transplanted with human CD34⁺ CB cells treated with or without human ANG (300 ng/ml) for 2 hr. BM was harvested 16 weeks post-transplant (n = 9–10).

(D) LT-HSC frequencies (black line) and 95% confidence intervals (shaded boxes) for each transplant condition from Figure 7C ($p = 8.28 \times 10^{-5}$).

(E) Secondary transplant of ANG-treated human CD34⁺ CB cells from primary recipient. BM was harvested 4 weeks post-transplant (n = 6).

Error bars indicate mean ± SEM, and heatmaps represent mean. *$p < 0.05$; **$p < 0.01$; ns, not significant.

See also Figure S7.

et al., 2010; North et al., 2007); however, it has been noted that cycling HSPCs engraft less well upon transplantation and undergo faster exhaustion (Nakamura-Ishizu et al., 2014; Passegué et al., 2005), likely as a consequence of differentiation and loss of stemness. Our finding that ANG improves HSPC stemness warrants further testing of ANG as a means of improving transplantation outcomes in the setting of limiting HSPC cell numbers. Further, the ability of ANG to serve as a radio-mitigant is also of considerable interest, particularly given its ability to rescue animals when administered 24 hr post-irradiation injury. Translation of this ability to humans is obviously complex, but the potential to reduce mortality following radiation exposure is of considerable significance. Functionally, we demonstrate that ANG simultaneously preserves stemness and promotes progenitor cell proliferation following radiation damage, in contrast to other reported approaches of HSPC regeneration or protection from genotoxic injury. The success of hematopoietic regeneration depends upon rapid reconstitution of mature blood cell pools, to avoid infections and bleeding complications, and long-term generation of mature cells from a durable cell source (Doulatov et al., 2012; Smith and Wagner, 2009). These two functions are provided by progenitor and stem cell populations, respectively.

Currently, there are no FDA (U.S. Food and Drug Administration)-approved drugs to treat severely irradiated individuals (Singh et al., 2015). A number of hematopoietic growth factors have been shown to mitigate hematopoietic syndrome of acute radiation syndrome; however, only a few candidates have been demonstrated to improve survival when administered 24 hr post-irradiation (Himburg et al., 2014), an efficacy requirement mandated by The Radiation and Nuclear Countermeasures Program at the National Institute of Allergy and Infectious Diseases. Moreover, current standard-of-care approaches, including granulocyte colony-stimulating factor (G-CSF) and its derivatives, target a limited progenitor cell pool and require repeated doses to combat radiation-induced neutropenia (Singh et al., 2015). In this regard, ANG is a promising candidate as a medical countermeasure for radiation exposure, as only three ANG treatments are needed for improved animal survival, even if started 24 hr after a lethal (12.0-Gy) dose. The long-term effect of ANG treatment in post-irradiated mice, however, is not clear and is the subject of future studies. It will, therefore, be important to assess whether ANG promotes the survival of genetically aberrant HSPCs and/or leads to development of leukemia.

Overall, we demonstrate that the unique growth and survival properties of ANG in primitive hematopoietic cells can be therapeutically harnessed for improvement of tissue regeneration.

EXPERIMENTAL PROCEDURES

Animal Studies

Ang$^{-/-}$ mice were generated in house. B6.SJL and NSG mice were purchased from The Jackson Laboratory. For aged-animal experiments, 22-month-old WT (NIH/NIA [National Institute on Aging]) and *Ang*$^{-/-}$ mice were used. For all other studies, age-matched 7- to 12-week-old mice were used. Littermates and gender-matched animals were used whenever possible. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Tufts University/Tufts Medical Center.

Cell-Surface Markers for Stem and Progenitor Subtypes

The following cell-surface markers were used: LKS (Lin$^-$c-Kit$^+$Sca1$^+$), MyePro (Lin$^-$c-Kit$^+$Sca1$^-$), LT-HSC (Flk2$^-$CD34$^-$ LKS or CD150$^+$C48$^-$ LKS), ST-HSC (Flk2$^-$CD34$^+$ LKS or CD150$^+$C48$^+$ LKS), MPP (Flk2$^+$CD34$^+$ LKS or CD150$^-$C48$^+$ LKS), MPP1 (CD150$^+$CD48$^-$CD135$^-$CD34$^+$ LKS), MPP2 (CD150$^+$CD48$^+$CD135$^-$CD34$^+$ LKS), MPP3 (CD150$^-$CD48$^+$CD135$^-$CD34$^+$ LKS), MPP4 (CD150$^-$CD48$^+$CD135$^+$CD34$^+$ LKS), CLP (Lin$^-$c-Kit^{med}Sca1^{med}IL7R$^+$ Flk2$^+$ B220$^-$), pre-pro B (Lin$^-$c-Kit^{med}Sca1^{med}IL7R$^+$ Flk2$^+$ B220$^+$), CMP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^+$CD16/32$^-$), GMP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^+$CD16/32$^+$), and MEP (Lin$^-$c-Kit$^+$Sca1$^-$CD34$^-$CD16/32$^-$).

Stem Cell Cultures

Mouse LT-HSCs were cultured per manufacturer's instructions in PBS or in S-clone media (Sanko Junyaku) for 2 hr or 2–14 days, respectively. Human CD34$^+$ CB cells were cultured per manufacturer's instructions in PBS or in StemSpan SFEM (serum-free expansion medium) for 2 hr or 7 days, respectively.

Transplantation

Transplantation of conditional knockout donor cells (1:1 competitive), transplantation of B6.SJL donor BM into conditional knockout recipients, ex vivo reconstitution assays, serial and rescue cells, tiRNA-transfected donor cells, irradiated or 5-FU-treated donor cells, and treated human CD34$^+$ CB cells were performed as described in the Supplemental Experimental Procedures.

Statistical Analyses

All bar graphs represent mean ± SEM, and all heatmaps represent mean. All data are derived from two to four independent experiments. For comparisons of two experimental groups, an unpaired two-tailed Student's t test was used (Excel). Kaplan-Meier survival curves were analyzed by log rank tests (Prism 6). Heatmaps were generated with RStudio. Limiting dilution analysis (LDA) was assessed by ELDA (http://bioinf.wehi.edu.au/software/elda). For all analyses, p values are given in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.042.

AUTHOR CONTRIBUTIONS

K.A.G., L.S., D.T.S., and G.H. conceived the project, designed experiments, and analyzed data. K.A.G., L.S., S.L., N.S., M.G.H., and H.Y. performed experiments. K.A.G., D.T.S., and G.H. wrote the manuscript.

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Tufts Medical Center researchers find new functions of blood cell protein in transplant

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BOSTON (August 11) - Tufts Medical Center and Tufts University scientists have found exciting, new functions of the protein angiogenin (ANG) that play a significant role in the regulation of blood cell formation, important in bone marrow transplantation and recovery from radiation-induced bone marrow failure. Since current bone marrow transplantations have significant limitations, these discoveries may lead to important therapeutic interventions to help improve the effectiveness of these treatments. The findings were published in an article, "Angiogenin promotes hematopoietic regeneration by dichotomously regulating quiescence of stem and progenitor cells," in the August 11, 2016 issue of the journal *Cell*.

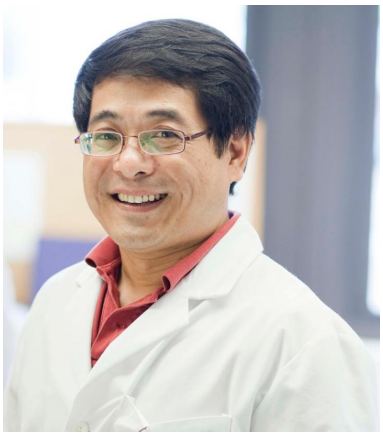

IMAGE: GUO-FU HU, PHD, IS AN INVESTIGATOR IN THE MOLECULAR ONCOLOGY RESEARCH INSTITUTE AT TUFTS MEDICAL CENTER **view more** ›

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In the paper, the researchers show for the first time that ANG simultaneously reduces proliferation of stem cells and promotes proliferation of myeloid progenitor cells that give rise to mature myeloid cells. They further report that these two-pronged processes are accomplished by a novel molecular regulating mechanism, a first-ever such finding.

These findings have significant implications for both human stem cell transplantation and for radiation exposure. Cancer patients undergoing stem cell transplantation face two hurdles: the short-term challenge of having enough white blood cells to fight possible infections immediately following the transplant and the long-term challenge of sustaining stem cell function to maintain immunity. People exposed to large doses of radiation face challenges due to bone marrow failure induced by such exposures.

"We knew that ANG was involved in promoting cell growth so it was not unexpected to find that ANG stimulates proliferation of myeloid progenitor cells," said Guo-fu Hu, PhD, Investigator in the Molecular Oncology Research Institute at Tufts Medical Center, and the paper's senior author. "But it was surprising to find that ANG also suppresses growth of stem cells and that it accomplishes these divergent promotion or suppression functions through RNA processing events specific to individual cell types. Our discoveries suggest considerable therapeutic potential."

Dr. Hu also serves as faculty in the Biochemistry; Cell, Molecular & Developmental Biology; and Cellular & Molecular Physiology programs at the Sackler School of Graduate Biomedical Sciences at Tufts.

In a series of experiments, the team from Tufts MC and the Sackler School at Tufts, which collaborated with scientists at Massachusetts General Hospital, isolated and described the divergent regulatory functions of ANG. They demonstrated how ANG stimulates proliferation of myeloid progenitor cells. They showed how ANG maintains stem cells by inducing a state of quiescence, or cellular dormancy, the first known evidence of ANG's suppressive activity. Quiescence preserves stem cells over time so that they will be available in the future to help maintain immunity.

In another novel finding, the team demonstrated that ANG achieves these dual functions by inducing RNA processing that is different in various cell types. In hematopoietic stem/progenitor cells, ANG induces processing of a specific type of RNA (tiRNA) that is quiescence-related whereas in myeloid progenitor cells, ANG induces processing of a specific type of RNA (rRNA) that is proliferation related. tiRNA is a type of small RNA that suppresses global protein synthesis, while rRNA or ribosomal RNA is a type of RNA molecule that enhances protein synthesis.

"Proper blood cell production is dependent on functioning hematopoietic stem and progenitor cells that are destroyed during conditioning procedures for transplantation or following bone marrow injury," said the study's first author Kevin A. Goncalves, who performed this research as part of his PhD studies in cellular and molecular physiology at the Sackler School. "Our study demonstrates that ANG regulates critical functions of both clinically-relevant cell types."

In further studies, the researchers tested the capacity of ANG to prevent and mitigate radiation-induced bone marrow failure, and in pre-clinical models, they found that survival following radiation exposure was increased after treatment with recombinant ANG protein.

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A complementary paper, "Proximity-Based Differential Single-Cell Analysis of the Niche to Identify Stem/Progenitor Cell Regulators," published online on August 11, 2016 in the journal *Cell Stem Cell*, reports the discovery and confirmation of ANG as a niche regulator.

Additional authors are Shuping Li, MD, PhD, Miaofen G. Hu, MD, PhD from Tufts Medical Center; Hailing Yang, PhD, a recent graduate of the Sackler School; and Lev Silberstein, PhD and Nicolas Severe, PhD from Massachusetts General Hospital and Harvard University. David Scadden, MD, also from Massachusetts General Hospital and Harvard University is co-corresponding author.

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About Tufts Medical Center and Floating Hospital for Children

Tufts Medical Center is an exceptional, not-for-profit, 415-bed academic medical center that is home to both a full-service hospital for adults and Floating Hospital for Children. Conveniently located in downtown Boston, the Medical Center is the principal teaching hospital for Tufts

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Tufts University School of Medicine and the Sackler School of Graduate Biomedical Sciences are international leaders in medical and population health education and advanced research. Tufts University School of Medicine emphasizes rigorous fundamentals in a dynamic learning environment to educate physicians, scientists, and public health professionals to become leaders in their fields. The School of Medicine and the Sackler School are renowned for excellence in education in general medicine, the biomedical sciences, and public health, as well as for innovative research at the cellular, molecular, and population health level. The School of Medicine is affiliated with six major teaching hospitals and more than 30 health care facilities. Tufts University School of Medicine and the Sackler School undertake research that is consistently rated among the highest in the nation for its effect on the advancement of medical and prevention science.